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# A Field Attempt to Assess the Mating Competitiveness of Sterile Males Produced by Crossing 2 Member Species of the *Anopheles gambiae* Complex

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*In laboratory experiments sterile males produced by crossing member species of the* Anopheles gambiae *complex competed very successfully with normal males for normal females and the latter when mated with sterile males laid sterile eggs.*

*A field trial was arranged in a small isolated village near Bobo-Dioulasso, Upper Volta; the trial took place at the end of the rainy season against a declining population of species A. Sterile males were produced from the cross between species B males and* An. melas *females; this cross yielded an $F_1$ generation mainly composed of sterile males with a sporadic occurrence of a sometimes significant proportion of females which were reproductively normal.*

*The $F_1$ generation was reared to pupae and these were released into existing breeding places and later from artificial containers.*

*Some 300 000 pupae were so released over a period of 2 months and adult collections were made periodically from inside houses and from outside shelters after releases started. Of the males caught, 75 % proved to be sterile while nearly 6 % of normal-looking ovipositions failed to hatch. However 2.5 % of them proved to be from hybrid females and not from species A females. In control villages, 1.35 % of normal-looking ovipositions did not hatch.*

*It was concluded from this field trial that the sterile males were not mating on any significant scale with the natural species A females. This could have been due to a number of factors but the most important is considered to be an ethological one—a mating barrier preventing mating between introduced sterile males and natural females. This is strongly expressed under natural conditions but not operative in the limited confines of a cage. The use of a cross between two species against a third species may well have enhanced this barrier.*

Laboratory cage experiments carried out in London with sterile males produced by crossing various member species of the *Anopheles gambiae* complex showed conclusively that when sterile males are released in low proportion to normal males they still succeeded in mating with females and that these females laid infertile eggs (WHO Scientific Group on the Genetics of Vectors and Insecticide Resistance, 1964; Davidson, 1964, 1969a, 1969b; Davidson et al., 1967). It was further shown that these females, even when subsequently forcibly mated with normal males, continued to lay sterile eggs (Bryan, 1968). The latest laboratory experiments (Davidson, 1969b) made use of those crosses which produced a high proportion of sterile male offspring and very few females (which are known to be fertile), namely, crosses between the males of species A and B and the females of *An. melas* and *An. merus*. First-instar larvae from such crosses were added in various proportions to breeding bowls containing normal first-instar larvae and the mixed larvae were reared together to the adult stage. The adults were then caged together and the number of females that subsequently laid sterile eggs was recorded.

When these experiments were being carried out, it was known that there was a possibility of a field trial of the technique in the village of Pala near Bobo-

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Dioulasso, Upper Volta. Thus a colony of species A from this village was used in London to cross with *An. melas* and the $F_1$ sterile male was tested against the Pala species A strain. It was noted in the course of these experiments that this cross, Pala species A male × *An. melas* female, consistently gave high proportions of female offspring, and that the number of females that laid sterile eggs, from cages containing mixed normal and sterile males, was considerably less than when a cross between the males of a species B strain from Kano, Nigeria, and females *A. melas* was used against the same species A strain from Pala. This latter cross, between Kano species B males and *An. melas* females resulted in far fewer females in the $F_1$ generation. It was thus decided to use this cross in the field trial in Upper Volta even though it was realized that a cross between 2 species was being used to attempt to control a third species. Hybrid vigour was considered to be an important characteristic of the sterile male and perhaps it would prove sufficient to overcome the natural mating barrier between species just as successfully as it had in cages. This then was the prime purpose of the field trial, to find out if these sterile males would succeed in mating under natural field conditions with the females of a natural population.

The existence of crosses producing almost nothing but sterile males suggested a simple way of introducing these sterile males into a natural population, namely, by introducing them at the stage when the $F_1$ eggs are about to hatch into the few, permanent, identified, breeding places remaining in the dry season. The larvae destined to produce sterile males would then be expected to develop alongside the natural larvae under the same breeding conditions and the adult sterile males would be expected to emerge at the same time and at the same place as the normal males and females. It was with this idea in mind that computer calculations were made (Cuellar, 1969a) of the most favourable ratio of sterile-male-producing eggs to normal eggs required to produce eradication in a comparatively short space of time. This ratio was shown to be 2 : 1 (allowing for the fact that only half the hybrid eggs hatch) and if it were maintained over a period of approximately 9 weeks eradication would be expected, provided that the sterile males were equally good at mating and that no female lived longer than 9 weeks. These calculations took into account that the mortality occurring between the egg stage and adult emergence under field conditions must be exceedingly high to maintain a static population (more than 98%) and that the continuous addition of the sterile-male-producing eggs would ensure a continuing high mortality of the aquatic stages.

The successful application of this method in the field would then depend on knowing all the breeding places and on having some method of estimating the natural population size. On a limited scale this would mean a small number of breeding places and in practice would indicate that the method should be applied in the dry season, preferably at the beginning, so that introductions of sterile males would be into a declining population. These conditions were found to exist during a visit to the village of Pala in Upper Volta in October 1967.

In October 1968, however, when the field trial was initiated, conditions were very different. Unusually late rains continued into the beginning of November, and resulted in the presence of numerous, ill-defined, breeding places and it was not until the end of that month that conditions approached those existing in October 1967. Attempts made in October 1968 to estimate first-instar larval population sizes by the mark–release–recapture method, using larvae stained with Giemsa, were of limited success. Laboratory trials with this stain had shown that the blue coloration produced was persistent, was easily recognized and had little adverse effect on the larvae. However, the number of small well-defined pools in which the method would have been expected to yield results of some accuracy were few and certainly not representative of the majority of breeding places. In fact much of the breeding was at the edges of small rivers and irrigation ditches and in quiet back-waters surrounding the village. Sample dips made in the first half of October 1968 from different breeding sites near the town of Bobo-Dioulasso, in the village of Pala itself and in the 2 control villages, Koro and Borodougou showed, in most cases, the presence of low proportions of advanced larval stages and very small proportions of pupae, confirming a high mortality in the aquatic stages (Table 1). Only 1% of more than 6000 aquatic stages counted were pupae. Not all the breeding places were the same in this respect, however. Temporary rain-water pools at Kua, for example, showed higher proportions of late larval and pupal stages. In Pala itself, in November, when breeding places were rapidly drying-up, one pool (pool M in the accompanying map) was sampled over the 8 days before it dried up completely and it showed very intensive breeding; 80% of the material counted from this pool was in

TABLE 1

DISTRIBUTION OF AQUATIC STAGES IN BREEDING PLACES IN THE VICINITY OF BOBO-DIOULASSO IN OCTOBER AND NOVEMBER 1968

| Stage | Locality and dates | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Bobo-Dioulasso town | | Bobo-Dioulasso outskirts | | Kua | | Pala | | Koro | | Borodougou | | Total | | Pala "M"[a] | |
| | 9-10 Oct. | | 11-15 Oct. | | 3-5 Oct. | | 8-19 Oct. | | 7-14 Oct. | | 7-11 Oct. | | 3-19 Oct. | | 20-27 Oct. | |
| | No. | % | No. | % | No. | % | No. | % | No. | % | No. | % | No. | % | No. | % |
| 1st instar | 96 | 39 | 1 264 | 66 | 195 | 50 | 747 | 47 | 111 | 58 | 1 412 | 70 | 3 825 | 61 | 5 | <1 |
| 2nd instar | 50 | 20 | 407 | 21 | 50 | 13 | 322 | 21 | 41 | 21 | 429 | 21 | 1 299 | 20 | 39 | 2 |
| 3rd instar | 55 | 22 | 164 | 9 | 55 | 14 | 275 | 18 | 29 | 15 | 105 | 5 | 683 | 11 | 328 | 18 |
| 4th instar | 44 | 18 | 56 | 3 | 70 | 18 | 211 | 14 | 11 | 6 | 59 | 3 | 451 | 7 | 1 085 | 62 |
| Pupae | 3 | 1 | 14 | 1 | 22 | 5 | 5 | <1 | 0 | 0 | 16 | 1 | 60 | 1 | 312 | 18 |

[a] Pool M on the map.

the fourth-instar and pupal stages. This represented a concentration of breeding from a quite extensive river into a pool rapidly decreasing in size and to which little in the way of new individuals was being added.

To gain some idea of the survival of the sterile-male-producing larvae of the hybrid generation in a natural breeding place, 700 of them were added in various larval stages over several days (all those available at the time) to a large pool near Bobo-Dioulasso estimated by the mark–release–recapture method to contain some 21 000 first-instar larvae. Samples of larvae and pupae were taken on successive days after this introduction and were reared to the adult stage in the laboratory. Emerging males were then dissected to see how many were sterile. Only 1 sterile male was recovered out of 86 dissected. The rough way in which this experiment was carried out did not enable any accurate predictions to be made of expected proportions of sterile to normal males emerging from this breeding place but the detection of only 1 sterile male after 700 had been introduced did indicate an exceedingly high mortality.

As well as being presented with a larger number of breeding places than originally expected, many of them ill-defined, and being faced with the impossibility of assessing larval population sizes with any degree of accuracy, logistic considerations also had to be taken into account in planning the field trial. According to Cuellar (1969a) 2 000 000 hybrid eggs a day would be required to control a daily emerging population of 10 000 males and females while the release of 300 000 adult sterile males would achieve the same object against the same population size, but after 13 weeks instead of 9 (Cuellar, 1969b). With the time and facilities available it was decided that there would be more likelihood of achieving the required number of adult sterile males than the required number of eggs and thus the $F_1$ generation was reared under ideal conditions in the laboratory to the pupal stage and with only a moderate aquatic-stage mortality. The pupae were then to be taken to the village of Pala and there released.

## THE PROJECT AREA

The village of Pala near the town of Bobo-Dioulasso in Upper Volta was selected as the site for sterile-male releases because of its small size, its relative isolation and its proximity to the laboratory and insectary facilities of the Entomology Section of the Centre Muraz (Office de la Recherche Scientifique et Technique Outre-Mer). These laboratories also serve as a WHO International Reference Centre under Dr J. Hamon, who acts as the principal investigator. In addition, the village is well known entomologically and has served as a site for numerous investigations by French research workers in the past.

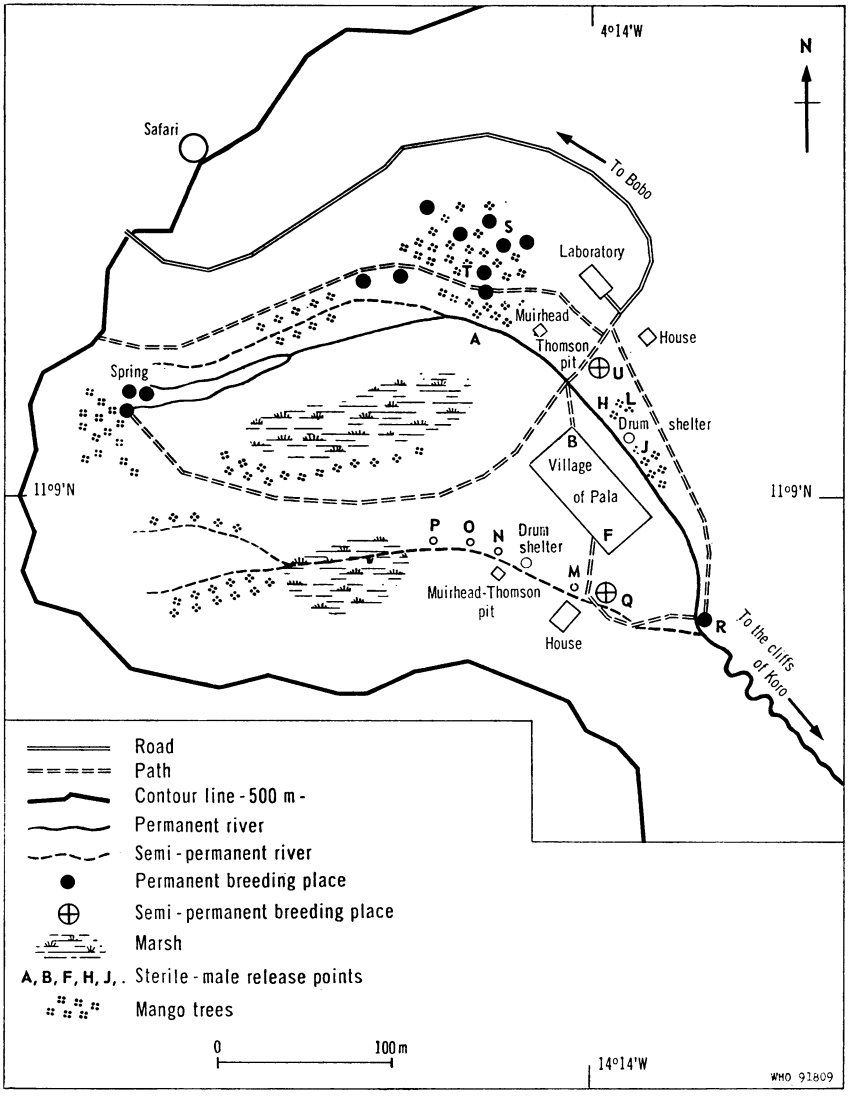
Close to it are 2 other similarly isolated villages, Koro and Borodougou, which served as controls.

Actual distances "as the crow flies" between these villages and the town of Bobo-Dioulasso are:

| | |
|---|---|
| Bobo-Dioulasso centre to Pala | 7 km |
| Pala to Koro | 4 km |
| Koro to Borodougou | 4 km |
| Pala to Borodougou | 7 km |

It was considered important that the village in which the release of sterile males was to be made was sufficiently isolated that little or no immigration of mosquitos from surrounding areas could occur, that, in fact, any effect of sterile-male mating with local natural females would not be masked by a large immigration of fertilized females from outside the treated area. A survey of the village of Pala and its surroundings did reveal that virtually all breeding was in the immediate vicinity of habitations and the immediate surrounding cultivated land, but at a distance of 2 km to the east and west were 2 permanent rivers which might have been sources of

PALA VILLAGE AND SURROUNDINGS



additional breeding. However, a limited mark–release–recapture experiment indicated that very few mosquitos would be expected to invade the village from them.

Between 5 November and 9 November the insecticide Abate was applied to the 2 rivers and their associated pools. A water fall on the river east of Pala was used as a source for an application of 1 ppm for 30 minutes and evidence of its efficiency was given by the disappearance of *Simulium* larvae for a distance of 1.5 km from the point of application. Other places were treated at a rate of 560 mg of active product per 10 m². This " insecticidal barrier " helped to ensure the isolation of the village of Pala.

Pala is a small village of some 500 inhabitants housed in dwellings with mud walls and mud ceilings mostly joined to one another. The total number of rooms in the village was estimated to be in the region of 311. A small permanent river arises from underground springs to the west of village and flows close to the north-east border of the village: this river and a few permanent pools to the north and south-east form the main breeding areas of *An. gambiae* in the dry season. In the wet season a small temporary river runs to the south of the village to join the permanent one south-east of the village and in addition numerous small rainwater pools occur all around the village. *An. gambiae* bred in all these places in the rainy season. The accompanying map shows the village and the surrounding area and pin-points the actual sites where sterile-male releases were made. To the north of the village a small house with mud walls and a thatched roof served as a field laboratory and female *An. gambiae* caught in houses in the village (and in the control villages) were kept there for oviposition.

It is almost certain that the *An. gambiae* population in Pala is entirely composed of species A. More than 40 identifications have been made from this village over the past 10 years. Species B was found in 1958 and again in 1965 and 1966 but since then only species A has been identified: 18 of these identifications were made just prior to the present field trial. Species A was identified 5 times from the control village of Borodougou in the present investigation.

The control village of Borodougou resembled Pala in many ways with permanent water in evidence close to the village in the dry season. Koro differed in that it was on top of a small hill, had less permanent water and was some distance from the village.

COLONIES AND CROSS-PRODUCTION

The 2 species of the *An. gambiae* complex used to produce sterile males were species B and *An. melas*. The species B strain was a dieldrin-resistant one originating from Kano, Nigeria, and started as a colony in the Ross Institute in London in 1958. The *An. melas* strain had its origins near Harbel, Liberia, was started as a colony in the Harbel Institute for Medical Research prior to 1962, and was subcultured in the Ross Institute in London in 1962.

Both strains were sent to Bobo-Dioulasso from London by air in the egg stage. Little difficulty was experienced in starting the species B colony in the insectaries of the Centre Muraz but considerable difficulty was experienced in establishing *An. melas*. Though numerous consignments of this species were despatched from as early as January 1968 it was only in July that a self-perpetuating colony was eventually started from a comparatively small number of individuals, presumably through some process of selection of a few mosquitos adapted to the particular conditions of the Centre Muraz insectaries. It was not until the middle of October, however, that the *An. melas* production was high enough to start crossing to produce sterile males.

The high chlorine content of the town water supply ruled out its use for rearing larvae. In its place distilled water, with sufficient sea-salt added to give 20% sea water (7 g per litre), was used for rearing *An. melas* and river water from the river east of Pala, north of the point of introduction of the insecticide Abate, was used for rearing the species-B colony and the cross. Rearing was carried out in rectangular enamel containers of various sizes with strict attention to particular densities for particular surface areas, especially in the case of *An. melas*. Here 1 larva per 1.5 in² (or about 1 larva per 10 cm²) was aimed at. Hybrid larvae were reared in higher density at approximately 1 per 1 in² (6.5 cm²).

For the first 24 hours of larval life a small quantity of spinach juice, produced by pounding spinach leaves, was given and thereafter finely ground Farex (a proprietary cereal baby-food) was sprinkled on to the water surface in greater and greater quantity and at more frequent intervals as the larvae grew in size. The aim was to add more food as soon as, or shortly after, the previous food had been removed from the water surface. A change of water when the larvae reached the fourth instar proved advan-

tageous in the case of *An. melas* but was not usually necessary with species B or the cross. Pupation usually started on the eighth day after the hatching of the eggs.

Pupae were collected by hand and counted into lots of 600 and each lot was allowed to emerge into a cage 30 cm × 30 cm × 30 cm. In each cage 5% sucrose solution was provided and was changed every 2 days. The feeding of the females was on immobilized rabbits laid stomach downwards on the top of the cage. Rabbits were the only suitable small mammals readily available but were on the whole unsatisfactory, being subject to a high handling mortality and being excessively expensive. The species B colony readily laid eggs on damp filter-paper but the *An. melas* colony and the cross oviposited better on free water in glass dishes lined with filter-paper to prevent the stranding and desiccation of the eggs. All eggs were kept on damp filter-paper for 1 day before being introduced into the larval-rearing containers.

The capacity of the insectary was such that approximately 3000 *An. melas* and 2600 species B pupae could be produced each day. Of the 3000 *An. melas* pupae, 600 were used for colony maintenance while the other 2400 were set aside for emergence and for the isolation of approximately 1200 virgin females (removed within about 12 hours of emergence) for the cross. Of the 2600 species B pupae, 200 were used for colony maintenance while the other 2400 were used as the source of approximately 1200 males for the cross. The cross between species B males and *An. melas* females was then made in cages of 300 males and 300 females. Thus at any one time 7 cages of *An. melas*, 3 cages of species B and 28 cages of crosses were in existence producing on average of something like 6000 *An. melas*, 4000 species B and 40000 hybrid eggs per day. Approximately 300 enamel containers with an average surface area of 500 $in^2$ were needed to rear these aquatic stages. As on average less than 50% of the hybrid eggs hatched (those destined to become sterile males; only very few females normally resulted from this cross) a maximum yield of hybrid pupae of approximately 15 000 per day was expected, allowing for some aquatic-stage mortality. In fact this number was never reached; the maximum was 12 000 on one day and the average at the height of production was only about half this figure.

For some unknown reason the proportion of females in the hybrid generation produced from the cross, species B males × *An. melas* females, fluctuated from a very low level to as much as 25%. This gave cause for concern, as these hybrid females are capable of producing offspring if mated by normal males. In terms of the present experiment they could have mated with the natural species A males present in the village of Pala. The result of such mating would be the production of a triple hybrid generation the sex ratio of which would be expected to be normal but the males of which would again be sterile. Such a triple hybrid generation was purposely produced in the laboratory by caging hybrid females with males of an existing self-perpetuating colony of species A derived from the village of Pala and maintained over many generations by the Centre Muraz staff. The offspring did in fact show a normal sex ratio with all the males sterile, but the females remained fertile and could be again successfully crossed to species A males.

Of equal concern was the possibility that these hybrid females in the field would " take up " sterile males which might otherwise mate with the females of the natural species A population. Evidence was found that this was in fact occurring. Fortunately the eggs of hybrid females could be distinguished from species A eggs. They resembled those of the mother species *An. melas*, having a broad deck and no significant gap between the dorsal edge of the float and the frill of the deck. *An. gambiae* species A eggs usually have a narrow, waisted deck and a considerable lateral gap between float and frill. Some of the eggs laid by females, caught in houses in Pala after sterile-male releases were made, were of the hybrid type. None hatched, however, and it is concluded that the mating, natural species A males × hybrid females, never took place but that matings between the sterile males and hybrid females did occur occasionally.

Considerable time and effort had been devoted in London to an investigation of the reason for the appearance of females in the hybrid generation. A number of single families of both parent strains (species B and *An. melas*) were raised and crossed individually in an attempt to find 2 families which when crossed would consistently give very high proportions of male offspring. Initial apparent success in this search was followed by a later reversion to the appearance of high proportions of females and the problem remains unsolved.

The species B strain from Kano used in these investigations had been selected in the past for the marker mutant *Black-diamond* (Mason, 1967). This appears to be a single, autosomal, dominant gene and

is expressed as a dark, diamond-shaped patch of pigment on the larval thorax. In the early larval instars it can be seen in both sexes. In the fourth larval instar, however, it is only expressed in the female and it disappears with larval growth in the male. Being dominant, it is also expressed in females of the hybrid generation. Thus its presence in fourth-stage larvae of the hybrid generation indicated the presence of females and a constant watch was kept for it. Where it was present in large numbers of larvae they were removed. Unfortunately the parent species B strain was not pure for the marker gene and a proportion of the females in both parent and hybrid generations were unmarked. Regular sampling of the sex ratio of the hybrid generation was done in addition to searching for marked larvae and the fluctuation in the percentage of females is given in Table 2. Regular dissections of hybrid males were also carried out to confirm sterility and to ensure that no mistakes had been made in the crossing procedure. No evidence of any mistake was ever found.

### RELEASES OF HYBRID PUPAE

Hybrid pupae were collected by hand and separated into lots estimated by experience to be between 500 and 1000. Counting as such was not done but an indirect measurement of quantity was used. A glass funnel 16 cm in diameter was graduated by pouring in lots of 100 pupae in an excess of water and running off the water until the pupae formed a continuous, uninterrupted film when at rest. If no shadow was cast and the water was allowed to run out very slowly an indication of the end-point was obtained when overcrowding caused sudden violent activity among the pupae. When graduation was completed the unknown quantities could be measured with a high degree of accuracy (see below).

For the transportation of the pupae from the laboratory to the village of Pala, a distance of 9 km by road, batches were filtered through gauze squares which were then laid on absorbent cotton-wool in 9-cm-diameter, plastic Petri dishes. Water was added from a pipette to float out the pupae into a single layer, after which the excess water was again removed with the pipette. Each container was labelled with the estimated number of pupae. The pupae were released in the field merely by removing the gauze from the Petri dish and floating off the pupae.

Throughout the period of pupal releases a check was kept on the accuracy of the funnel method of estimating pupal numbers, the sex ratio of emerging adults and the mortality occurring due to handling and transportation by allowing some of the batches of pupae to produce adults from artificial containers, usually glass Kilner jars placed in different positions in the village of Pala. These containers were collected on the day following adult emergence, and were taken back to the laboratory in Bobo-Dioulasso where the contents were carefully sorted and counted into pupal skins, dead pupae and dead adults. A sample of the pupal skins was then sexed by examination of the terminalia under a binocular dissecting microscope. This proved a very simple and rapid method of checking the sex ratio. The total number of pupal skins sexed in this way was 18 120 and just over 7% were female. The mortality determined from these jar-counts averaged 10% of 56 651 individuals counted and this latter number approached very closely the estimated number of 54 450 determined by funnel measurement.

The method and site of pupal release in the village of Pala was of necessity varied according to the quite dramatic climatic changes which occurred during the period from the end of October 1968 until the beginning of January 1969. Initially, on logical grounds, it was decided that the ideal release sites would be those breeding places that showed high larval densities with high proportions of advanced stages, this in itself indicating lack of predators. Around the village, 4 such places were found (A, L, M and N on the map) and hybrid pupae were released at these sites for about the first month, along with sample jars of pupae immersed in water, at the sites marked H, O and P (on the map), to give indications of mortalities (see above). Masses of pupal skins were recovered from the natural breeding places, thus indicating good adult emergence. From 24 November, however, a marked change in climate occurred with night air temperatures falling as low as 12°C. At the same time the natural breeding places used as points of release dried up; the last rainfall of the year was on 3 November. On the first of these cold nights, emergences from various types of artificial containers were tried both in empty houses in the village (B and F on the map) and immersed in the remaining water of the river at H and in a remaining pool at P (on the accompanying map). Considerable mortality occurred in all the containers at all the sites but particularly in aluminium pots where the heat exchange had been greatest. Clay pots and glass jars were more satisfactory but if they were

immersed in water outside it was essential that the water-levels inside and outside the container were the same. Where heat exchange had been great a prolongation of the pupal stage by several hours was evident and containers still with live pupae in them were left for a further day. All containers both inside houses and immersed in water had to be protected from animals, in particular goats, sheep, chickens and large lizards of the genus *Varanus*. This was done by covering them with chicken-wire netting, but this did not prevent the entry of small frogs into those containers immersed in the river and in pools.

By the end of November all that was left in the way of water in and around the village was the river, which incidentally was polluted in parts by the washing of clothes and cooking utensils by the villagers, and some permanent pools to the north and south-east. Light *An. gambiae* breeding was evident in some of these pools and as the water temperature remained above 20°C it was decided to release pupae in them although some risk of loss due to predators (frogs and fish) was inevitable. Thus releases were made at Q, R, S and T (on the map) from 29 November to 5 December. Pupal skins could be found in these pools after release but never in the large quantities found in the earlier releases. Because of this and because of the relative remoteness of most of the pools from the village it was eventually decided to make further releases from glass Kilner jars housed in a bed of straw inside 2 large mosquito cages (50 cm × 50 cm × 50 cm) placed at point J on the north-east side of the village on the edge of the river. The cages were large enough to hold 6–9 jars and the feet of the cages were immersed in water containers as a protection against invasion by ants. The mosquito-netting top of each cage was replaced by chicken-wire netting, the holes in which were large enough to allow escape of the sterile males while forming a protection against predators. All further releases until 3 January 1969 were made from these cages. Direct estimates of mortalities and sex ratios were made from sample jars on each day. This was considered to be the most satisfactory method of release under the prevailing climatic circumstances.

## POST-RELEASE OBSERVATIONS

Two kinds of catch were made by hand, at regular intervals after sterile-male releases started in the village of Pala, of mosquitos resting inside houses and of those resting outside in specially constructed shelters. Two types of shelter were tried; the conventional pit-shelter of the Muirhead-Thomson type and oil drums let into the banks of the rivers. The latter type yielded very little; most of the outside-resting mosquitos were derived from 2 Muirhead-Thomson pit-shelters situated on 2 sides of the village (see the map). In the control villages only house catches were made. House catches yielded both males and females. Pit-shelter catches gave mostly males and only an occasional female. Light traps were also tried both inside and outside houses but yielded only the occasional male and female *An. gambiae*.

The female *An. gambiae* caught were tubed in glass or plastic containers with netting tops through which water was added to a depth of 0.5 cm for oviposition. These tubes with females from Pala and from the control villages were kept in the field laboratory at Pala adequately protected against invasion by ants and covered to maintain a high humidity. Any females that did not develop eggs were dissected to find out whether they were fertilized or not and whether nulliparous or parous. Those dying in the gravid state without having oviposited were also dissected to see if they had spermatozoa in the spermatheca. Ovipositions were classified as normal in size and floating, or small and sunken. They were kept for several days if they did not hatch. Samples of all but the sunken eggs (which were difficult to identify) were examined whether they hatched or not to see if they were species A or hybrid. Females from Pala which oviposited were released in the field laboratory and the ovipositions from these females that hatched were returned to breeding places in the village.

Male *An. gambiae* were dissected and classified from the appearance of the testes as sterile or normal. The difference was very marked and could be seen under the high power of the binocular dissecting microscope after rupture of the testes with a needle. The normal showed the unwinding coils of mature spermatozoa: the abnormal showed the discrete spermatocytes (round cells) and immature spermatozoa.

## RESULTS

Table 2 sets out the detailed results in treated and control villages before and during approximately weekly periods after the release of sterile males. An unfortunate accidental contamination with insecticide of a part of the insectary in which the hybrid

TABLE 2

DETAILED RESULTS OF HAPPENINGS IN THE VILLAGE OF PALA AND IN 2 CONTROL VILLAGES BEFORE AND AFTER THE RELEASE OF STERILE MALES IN PALA

| Characteristic | Period | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Before sterile-male release | After sterile-male release | | | | | | | | | |
| | 28 Aug.-29 Oct. | 30 Oct.-9 Nov. | 10-16 Nov. | 17-23 Nov. | 24-30 Nov. | 1-7 Dec. | 8-14 Dec. | 15-21 Dec. | 22-28 Dec. | 29 Dec.-3 Jan. | 4-13 Jan. |
| **Pala** | | | | | | | | | | | |
| No. of pupae released | 0 | 5 463 | 22 730 | 34 900 | 51 670 | 52 580 | 49 580 | 39 320 | 22 060 | 17 510 | 0 |
| Mortality (%) | | | 5 (1 803) | 8 (10 175) | 10 (8 727) | 15 (6 633) | 16 (8 339) | 12 (7 779) | 12 (6 910) | 9 (6 285) | |
| Females (%) | 6 (1 866) | 1 (218) | 11 (1 284) | 11 (9 223) | 5 (3 620) | 5 (1 076) | 1 (2 217) | 3 (1 738) | 3 (1 200) | <1 (1 100) | |
| % Sterile males in houses | | 0 (12) | | 38 (24) | 44 (32) | 80 (25) | 82 (67) | 55 (22) | 85 (19) | 91 (32) | 62 (42) |
| % Sterile males in outside shelters | | | | 94 (28) | 88 (88) | 89 (27) | 100 (16) | 50 (2) | 100 (4) | 50 (2) | 50 (6) |
| No. of rooms searched | 113 | 24 | | 44 | 100 | 53 | 176 | 58 | 52 | 52 | 115 |
| No. of females per room | 3.3 | 6.8 | | 1.9 | 1.3 | 0.9 | 0.4 | 0.3 | 0.1 | 0 | 0.02 |
| No. of females ovipositing | 585 | 96 | | 61 | 89 | 35 | 60 | 10 | 4 | 0 | 2 |
| No. of ovipositions hatching | 581 | 92 | | 49 | 82 | 29 | 53 | 9 | 3 | 0 | 2 |
| Non-hatchers: | | | | | | | | | | | |
| Normal—species A | 2 | 1 | | 3 | 3 | 2 | 3 | 0 | 0 | 0 | 0 |
| Normal—hybrid | 0 | 0 | | 4 | 2 | 1 | 2 | 0 | 0 | 0 | 0 |
| Abnormal | 2 | 3 | | 5 | 2 | 3 | 2 | 1 | 1 | 0 | 0 |
| **Control villages** | | | | | | | | | | | |
| No. of rooms searched | 64 | 5 | 13 | 44 | 29 | 15 | 8 | 29 | | 14 | |
| No. of females per room | 11.3 | 20.4 | 4.5 | 1.6 | 1.5 | 4.3 | 5.8 | 2.1 | | 1.8 | |
| No. of females ovipositing | 364 | 54 | 40 | 41 | 33 | 39 | 27 | 41 | | 21 | |
| No. of ovipositions hatching | 357 | 45 | 37 | 39 | 32 | 37 | 27 | 39 | | 20 | |
| Non-hatchers: | | | | | | | | | | | |
| Normal—species A | 3 | 0 | 0 | 1 | 0 | 2 | 0 | 0 | | 1 | |
| Abnormal | 4 | 9 | 3 | 1 | 1 | 0 | 0 | 2 | | 0 | |

[a] The figures in parentheses are the actual numbers on which the percentages are based.

generation was being reared resulted in only very small releases in the first period from 30 October to 9 November. Peak production of about 7000 pupae a day occurred over the period from 24 November to 14 December. After this a decline occurred associated with the effect of the cooler weather lengthening the gonotrophic cycle of the adult female and the larval aquatic cycle. Releases ceased on 3 January 1969 when approximately 1000 larvae remaining in the rearing bowls were released in the permanent water remaining in Pala.

Mortalities, as estimated from sample containers, started off at the low level of 5% but increased to around 15% at the period of peak production which coincided with the advent of adverse weather conditions and the drying-up of the main breeding sites. A gradual improvement in mortalities in the latter part of December is attributed to the change in method of release to that from glass jars bedded in straw in large cages.

The percentage of females in the hybrid generation exceeded 10% at the beginning of significant releases but declined rapidly to negligible levels for the remainder of the release period.

That the sterile males were departing successfully from the release points and mixing in significant proportion with the natural *An. gambiae* population is shown by their capture both resting in houses and in the outside shelters. In fact from the middle of November onwards about 75% of the males caught were sterile ones; the proportion in outside shelters slightly exceeded that in houses.

Between mid-September and the end of October the number of female *An. gambiae* caught per room in the village of Pala averaged 6.5. An average of 9.9 females per room in September had declined to an average of 5.2 in October. The control village of Koro showed the slightly higher figure of 7.2 in the month of October while Borodougou, where most of the control collections were made, showed the significantly higher average of 14.9 in October. This latter control village showed densities consistently higher than Koro and Pala. When sterile male releases started on a small scale, the density in Pala was of the order of 7 female *An. gambiae* per room but this fell sharply in the middle of November to a figure of 2 per room and thereafter declined steadily until at the end of December no females were recovered by the hand-catching method. Apart from 2 caught on 7 January no further females were captured in this village though searches continued until 13 January. Control catches declined markedly until the end of November but then showed a temporary increase in the first half of December followed by a further fall in December, but even on 3 January nearly 2 females per room could still be found in Borodougou.

A total of 1804 female *An. gambiae* was tubed from the village of Pala before sterile-male releases were made and from the control villages of Koro and Borodougou over the whole period of the experiment. Of this total 70% laid eggs, exactly the same proportion as those tubed from Pala (numbering 502) after releases started. Of the latter, 23% died in the gravid state without laying; 4% of these appeared to be without spermatozoa in the spermatheca. No eggs developed in 7% of them; three-quarters of these were nulliparous and mostly without spermatozoa; a single parous female without spermatozoa could have been a hybrid or a Pala female mated with a sterile male. In the control villages over the same period of sterile-male releases, slightly higher percentages of females did not develop eggs or died in the gravid state without laying. Again most of the non-developers were nulliparous and a small percentage of the dead-gravids had no spermatozoa.

TABLE 3

PERCENTAGE OF FEMALE *AN. GAMBIAE* OVIPOSITING IN PALA AND THE CONTROL VILLAGES AND THE CONDITION OF THOSE NOT OVIPOSITING

| Characteristic | Pala [a] | Controls [a] |
|---|---|---|
| No. of females tubed | 502 | 616 |
| Percentage ovipositing | 70 (349)[b] | 65 (398)[b] |
| **Non-developers** | | |
| % Not developing eggs | 7 (36)[b] | 10 (61)[b] |
| % Nulliparous and sperm negative | 65 | 69 |
| % Nulliparous and sperm positive | 9 | 13 |
| % Parous and sperm negative | 3 | 0 |
| % Parous and sperm positive | 23 | 18 |
| **Dead and gravid** | | |
| % Dying gravid without laying | 23 (117)[b] | 25 (157)[b] |
| % Sperm negative | 4 | 3 |
| % Sperm positive | 96 | 97 |

[a] After sterile-male release in Pala and during the same period in the control villages.

[b] Figures in parentheses are the actual numbers counted.

All the parous non-developers were positive for spermatozoa. Details of these observations are given in Table 3.

Of 1245 layings from female *An. gambiae* caught in Pala before sterile-male releases and from the 2 control villages throughout the experiment, 1214 hatched; 22 of the 31 that did not hatch were considered as abnormal layings; only 2 of these abnormal layings came from Pala. After sterile-male releases, the percentage of abnormal layings was about the same in both Pala and the control villages (in the region of 5%). Of normal layings from female *An. gambiae* caught in Pala in the 2 ½ months over which sterile males were released 5.88% did not hatch. In the same period in the control villages only 1.35% of normal layings did not hatch. However, 2.52% of the normal layings from Pala proved to be from hybrid females and only 3.36% from species A females.

## DISCUSSION

An attempt has been made in Table 4 to simplify the detailed results of Table 2, to calculate an approximate total daily emergence of natural male and female *An. gambiae* in the village of Pala from the number of females caught per room and to relate these figures to the number of sterile males thought to be required from Cuellar's (1969b) estimation of 300 000 sterile males to eradicate a daily emerging population of 10 000 males and females in a period of 13 weeks. The figure for the actual number of sterile males released makes a generous allowance for mortality, before and at emergence, and for the emergence of some females.

It can be seen from these calculations that only from the second week in December did the number of sterile males released reach the estimated number required, though it must be emphasized that many

TABLE 4

PREDICTED AND ACTUAL HAPPENINGS IN THE VILLAGE OF PALA AND IN THE CONTROL VILLAGES AFTER THE RELEASE OF STERILE MALES IN PALA

| Period | Pala | | | | | | Control |
|---|---|---|---|---|---|---|---|
| | Females per room per day | Calculated daily emergence [a] (♂+♀) | Sterile males required per day [b] | Actual sterile males emerged per day [c] | % of males captured which were sterile [d] | Normal species A layings that did not hatch | Normal layings that did not hatch |
| 10–16 Nov. | 5 [e] | 2 400 | 72 000 | 2 600 | — | — | 0/40 |
| 17–23 Nov. | 1.9 | 912 | 28 800 | 4 000 | 68 | 3/61 | 1/41 |
| 24–30 Nov. | 1.3 | 624 | 18 720 | 5 900 | 76 | 3/89 | 0/33 |
| 1–7 Dec. | 0.9 | 434 | 14 400 | 6 000 | 85 | 2/35 | 2/39 |
| 8–14 Dec. | 0.4 | 192 | 5 760 | 5 700 | 86 | 3/60 | 0/27 |
| 15–21 Dec. | 0.3 | 144 | 4 320 | 4 500 | 54 | 0/10 | 0/41 |
| 22–28 Dec. | 0.1 | 48 | 1 440 | 2 500 | 87 | 0/4 | — |
| 29 Dec.–3 Jan. | 0 | ? | ? | 2 300 | 89 | — | 0/21 |
| 4–13 Jan. [f] | 0.02 | 10 | 300 | ? | 61 | 0/2 | — |

[a] No. of females per room caught by hand is estimated to be 50 % of those present and these represent 1/4 of the total allowing for 75 % exophily. It is assumed that there are 300 rooms in Pala.
'p' is reckoned as 0.9, the number of ovipositions taken as 3 and the time for these as 10 days.
Thus 5 per room = $\frac{5 \times 2 \times 4 \times 300}{10}$ = 1200 females: that is, 2400 mosquitos of both sexes per day.

[b] Based on Cuellar (1969b) who calculates that 300 000 sterile males per day will eradicate a population of 10 000 emerging adults of both sexes per day in 13 weeks.

[c] Calculated from daily average for that period less 20 % for mortality (varied from 5 %–16 %) and hybrid females (these varied from less than 1 % to 11 % but most of the time were less than 5 %).

[d] Captured both in houses and outside shelters.

[e] An estimate based on the figures of the previous week.

[f] Last pupal release was on 3 January 1969 but about 1000 larvae were also released and would be expected to produce a few males over the following days.

of the basic figures used in the calculations were to some extent inspired guesses. The proportion of normal species A ovipositions that did not hatch in the village of Pala, though it definitely exceeded that in the control villages, did not, however, show any definite sign of increase as the number of sterile males released approached and exceeded the numbers estimated to be required. The conclusion is reached, therefore, that the sterile males were not mating on any significant scale with the natural species A females. The sterile males were certainly present in high proportion, as judged from their capture both in houses and in outside resting places, and did mate with their own females as witnessed by the number of hybrid ovipositions that did not hatch. Of the 21 normal-looking ovipositions that did not hatch, 9 proved to be from hybrid females.

Other factors may also have contributed to this failure to produce significant numbers of sterile ovipositions. Low night temperatures may have affected the behaviour of the introduced sterile males, which, it may be remembered, were reared under artificial conditions on artificial food. Marked fluctuations in temperature were recorded in the enamel containers in which they were raised, from as low as 17°C to as high as 34°C on the same day. Fluctuations in natural breeding places were less. The lowest of the few water temperatures recorded was 19°C and the highest 24°C. Morning visits to release sites also showed the presence of emerged sterile males resting very close to and often still inside pupal containers, just above the water.

A further factor in the failure to produce sterile eggs may have been the use of a hybrid between 2 species to compete with a third. If some form of barrier exists that prevents the sibling species of the *An. gambiae* complex from crossing under natural conditions, and all available evidence points to the existence of a such a barrier (Paterson, 1967; Ramsdale, 1967), then the barrier between the elements of 2 species and a third must be equally, if not more effective. The fact remains, however, that such barriers are not effective under cage conditions and it was the primary purpose of this field trial to find out whether the same successful results attained in the laboratory cage would be achieved under natural conditions allowing a natural mating behaviour pattern. The answer from this trial would appear to be that a small amount of mating does occur between sterile males and natural females but that the proportion of sterile males required to produce any marked reduction in population size would be much higher than the calculations made by Cuellar (1969a, 1969b) had indicated. These calculations assumed an equal mating ability of sterile and normal male, as had been indicated from laboratory cage experiments.

### POSTSCRIPT

Dr C. B. Cuellar, with the aid of his computerized mathematical model (on which the original predictions of sterile-male requirements were made) has now carried out a detailed analysis of the results of the field trial described in this article. He finds that if, instead of considering the natural population as a static one, the actual enormous and rapid drop in population size is simulated and the different aquatic-stage survivals in temporary and permanent breeding places are taken into account, proportions of sterile layings comparable with those recorded can be reproduced if the sterile male is considered as one-half to one-eighth as competitive as the normal one. Furthermore, the model predicts that eradication would occur if such sterile males continue to be released at the same level, but that it would take longer than originally envisaged.

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## RÉSUMÉ

### ESSAI D'ÉVALUATION SUR LE TERRAIN DE L'APTITUDE À LA CONCURRENCE SEXUELLE DE MÂLES STÉRILES OBTENUS PAR CROISEMENT DE DEUX ESPÈCES APPARTENANT AU COMPLEXE *ANOPHELES GAMBIAE*

On a démontré de façon probante que dans les conditions d'encagement au laboratoire les mâles stériles produits par le croisement entre deux espèces du complexe *Anopheles gambiae* rivalisent avantageusement, sous le rapport de l'activité sexuelle, avec les mâles normaux; l'accouplement des femelles normales avec ces hybrides a pour conséquence la ponte d'œufs stériles. On a estimé nécessaire de vérifier si ces résultats intéressants se reproduiraient dans les conditions naturelles.

L'essai, qui a eu lieu à la fin de la saison des pluies dans un petit village isolé des environs de Bobo-Dioulasso (Haute-Volta), a été dirigé contre la population anophélienne locale appartenant à l'espèce A du complexe *An. gambiae*. Des croisements massifs, réalisés en insectarium, entre mâles de l'espèce B et femelles de l'espèce *An. melas* ont donné naissance à des générations $F_1$ quasi uniquement composées de mâles stériles, bien que, occasionnellement, on ait relevé une assez forte proportion de femelles à potentiel reproducteur normal. Il était prévu à l'origine que la libération des hybrides dans la nature et leur incorporation à la population anophélienne locale se ferait au stade de l'œuf, mais des impératifs techniques ont amené à poursuivre l'élevage jusqu'au stade de la nymphose.

Au total, quelque 300 000 nymphes stériles ont été libérées en 2 mois, après transport sur le lieu de l'essai; il est douteux cependant qu'elles aient été libérées en nombre suffisant pendant le 1er mois. Les insectes adultes ont été ensuite récoltés périodiquement dans les maisons et les abris extérieurs. La dissection des mâles a montré que 75% environ d'entre eux étaient stériles. Dans près de 6% des pontes apparemment normales, les œufs n'ont pas éclos, mais il est apparu que dans 2,5% des cas ils avaient été déposés par des femelles hybrides et non par des femelles de l'espèce A. Dans les villages voisins pris comme témoins, 1,35% des pontes apparemment normales n'ont fourni aucune descendance.

On peut invoquer diverses raisons pour expliquer l'échec de cette tentative d'élimination d'une espèce par la méthode des mâles stériles: libération d'un nombre trop restreint d'hybrides, durée insuffisante de l'essai, conditions climatiques défavorables, influence néfaste d'un élevage artificiel prolongé jusqu'au stade nymphal. Le facteur principal semble être néanmoins l'impuissance des mâles stériles à soutenir la concurrence sexuelle avec les mâles normaux dans les conditions naturelles. Le fait que les mâles hybrides utilisés pour l'élimination de l'espèce A ont été obtenus par croisement entre deux autres espèces a peut-être représenté un obstacle supplémentaire à leur accouplement avec les femelles de l'espèce anophélienne locale.


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